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Qualitative Determination of Enzymatic Degradation Products Obtained from Apple Cell-Wall Polysaccharides[1,2]

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Abstract. D-galacturonic acid, D-glucuronic, D-galactose, D-glucose, L-arabinose, D-xylose and several unidentified substances were found as the enzymatic degradation products of the polysaccharides in fruit of Golden Delicious, Stayman and York Imperial apple varieties.

Selective fractionation of alcohol-insoluble solids (AIS) into chemically pure polysaccharides was not possible by the techniques used. Pectic fractions were composed of uronides and to a lesser degree of pentosans and hexosans while hemicellulose and cellulose were composed of uronides and, to a greater degree of pentosans and hexosans.

The uronides of each fraction appeared to be a mixture of galacturonic acid and glucuronic acid. Difficulties in separating these uronides for identification were overcome by reducing the galacturonic and glucuronic acids to galactose and glucose. A degradation of the galactose and glucose gave pentose monomers.

Identification of both galacturonic and glucuronic acids in "pectic fractions" suggests that each must be measured when relating pectic substances to apple firmness.

INTRODUCTION

TEXTURE of both raw and processed food products is important to consumer acceptance. It is generally agreed that changes in the texture of fruits and vegetables are due to modifications of the cell-wall polysaccharides (3, 4, 5). These changes may manifest themselves as softening during maturation and storage and as a general break down during processing.

Considerable research has been conducted in an attempt to relate apple cell-wall components to firmness differences (1, 3, 5, 7). Deutel and Stutz (2) suggested that the softening of fruits during ripening and storage is due to the so-called protopectin-pectin transformation, but whether this is true, is still in question. Other workers have held that cellulose and possibly hemicellulose are also involved in the structural changes that take place in plant products. Most of the earlier findings were based on data obtained by extracting the polysaccharides from the plant tissue on a solubility basis, and then measuring. The extractants have been of different chemical compositions and concentrations; and extractions have been for varying times and temperatures.

In the present work the naturally occurring polysaccharides have been extracted into generally accepted fractions of apple alcohol-

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insoluble solids (AIS), and these fractions were then characterized by their monosaccharides.

The specific objectives of this study were: a) To fractionate the apple cell-wall complex into its naturally occurring polysaccharides by carefully standardized procedures; b) To further break down each fraction as completely as possible by enzymes into its respective monosaccharides; c) To separate and identify these enzymatic degradation products on a qualitative basis by the use of descending paper chromatography; d) To determine the effect of variety, maturation, and ripening on these degradation products.

Materials and Methods

Golden Delicious, Stayman, and York Imperial varieties were harvested at 4 stages of maturation during 1962. The apples were placed in 1° C storage and processed immediately after harvest and at approximately 25, 50, 75, and 100% of their expected storage life. On designated harvest and storage dates, 2-bushel samples were washed, peeled, cored, trimmed, and sliced. Firmness determinations were made on the sliced apples using the Lee-Kramer shear press (7).

At each sampling date, 500 g of ¼" thick apple slices, were placed in 900ml of boiling 95% ethanol for 3 minutes. Slices were removed, blended with 600ml of ethanol for 5 minutes and then sealed in cans for later analysis.

The contents of the cans were then thoroughly washed with 70% ethanol and the sugar free AIS dried in an 85° C oven. The AIS was ground in a Wiley mill through a 40 mesh screen and stored in glass bottles. To show the greatest difference in slice texture and in polysaccharide components, the prepared AIS of the early and late harvested apples were selected for particular emphasis in this work. The dates of picking, storage, and the codes for samples are given in Table 1.

Fractionation of Alcohol Insoluble Solids: The experimental procedure for the fractionation of apples AIS is shown in Fig. 1. Two g of AIS of each treatment were extracted with 100ml H_2O twice for 1½ hours at room temperature on a mechanical shaker. The H_2O soluble substances (Fraction 1) were set aside. The residue was then extracted with 35ml, 0.25% ammonium oxalate, 0.25% oxalic acid (AO-OA) 2 times for 1½ hours at 75° C. The residue was set aside (Fraction 4) with most of the pectic substances and hemicelluloses removed.

Starch Hydrolysis: After extraction with AO-OA and prior to addition of HCl the pH of the 4 fractions was adjusted to 6.0, 20 ml of a freshly made solution of 1% alpha and 1% beta amlyase were added, and allowed to stand 24 hours at 25° C to rid each fraction of starch.

HCl Precipitation: Forty ml of 0.1*N* HCl were added to the AO-OA soluble substances. The precipitate (Fraction 3) was later characterized as hemicellulose and was particularly heavy in early harvest

Table 1.—The dates of harvest, storage periods, and the codes for the Golden Delicious, Stayman, and York Imperial varieties.

Harvest		Storage periods		Codes
Dates (1962)	Codes	Days in 1°C	Codes	
		Golden Delicious		
Sept. 4	A	0	0	G0A
		50	2	G2A
		98	4	G4A
Oct. 11	D	1	0	G0D
		48	2	G2D
		96	4	G4D
		Stayman		
Sept. 17	A	0	0	S0A
		64	2	S2A
		127	4	S4A
Oct. 24	D	1	0	S0D
		71	2	S2D
		134	4	S4D
		York Imperial		
Sept. 25	A	0	0	Y0A
		69	2	Y2A
		134	4	Y4A
Nov. 5	D	1	0	Y0D
		66	2	Y2D
		135	4	Y4D

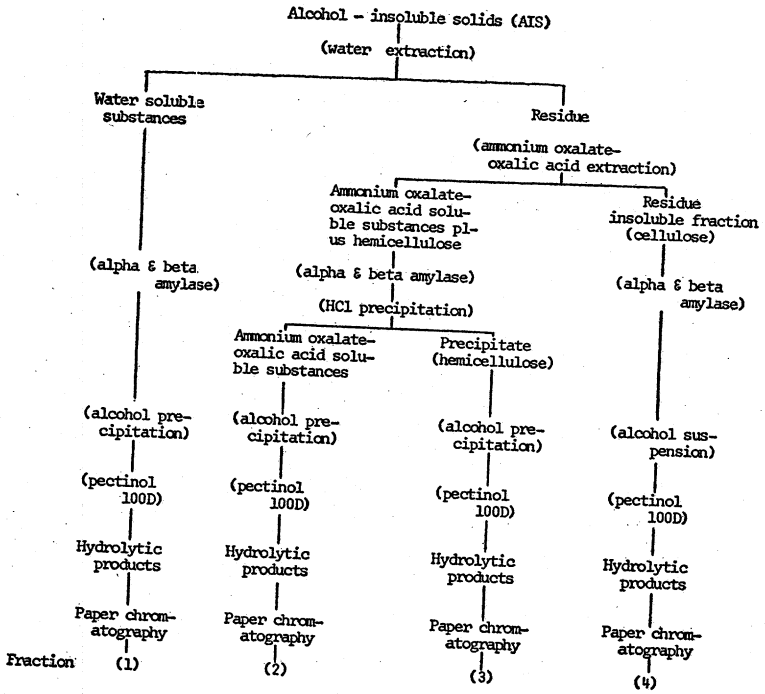


Fig. 1. Experimental procedure for fractionating of apple alcohol-insoluble solids.

fruit. The solute (Fraction 2) and the residue (Fraction 3) of this treatment were then separated by nylon cloth filtration.

Alcoholic Precipitation: To purify extracts, all 4 of the fractions were precipitated or suspended in 95% ethanol. The precipitate was allowed to stand overnight, and was then separated from the alcohol by filtering and washing on Whatman No. 1 filter paper.

Pectinol-100D[3] *Treatment of Fractions:* The pH of each fraction was adjusted to 5.5 for addition of freshly made 1% Pectinol-100D. The enzyme treated fractions were covered with 10 ml of toluene and allowed to stand 96 hours at 25° C. After this, the hydrolysates were filtered through Whatman No. 1 filter paper and the filtrate was placed in sample bottles and frozen.

Paper Chromatography: The enzyme treated extracts were applied to Whatman No. 3mm filter papers with a micro-pipette. They were irrigated with a mixture of normal amyl-alcohol, pyridine, and water (7-7-6v/v) in a sealed chromatographic cabinet. The papers were withdrawn after 34 hours at 25° C. After drying, the papers were sprayed with a location reagent, described by Partridge, (6) and prepared by mixing aniline (1 ml) and phthalic acid (1.6 g) with 100 ml of water saturated with butanol. The papers were dried and then heated in an oven at 100° C for 15 minutes. Color slides were prepared from the papers immediately after removal from the oven. Chromatograms were also prepared from standard reference sugars using 1% solutions of D-galacturonic acid, D-glucuronic acid, D-galactose, D-glucose, L-arabinose, and D-xylose. In addition chromatograms were prepared using filtered Pectinol-100D and distilled water. No color developed in columns which contained only the enzyme preparation and the distilled H_2O.

Two hydrolyzed fractions were selected for further study. They were degradation products of the AO-OA soluble material (Fraction 2) and the hemicellulose hydrolysate (Fraction 3) which had been precipitated by HCl. Concentrations were adjusted to give separate and compact spots with a satisfactory color selection. These papers were developed and then photographed in color.

Spectrophotometry: Although the study was basically one to characterize monosaccharides in extracted fractions, a pink colored spot which separated with the uronides was eluted off the paper chromatogram and studied by ultra-violet spectrophotometry.

Partial Reduction and Degradation of Hexuronides and Aldohexoses: Apple uronides were eluted from Whatman No. 3mm paper after 48 hours using the H_2O, amyl alcohol, pridine solvent. Sprayed marker strips indicated all sugars except uronide had dripped from the paper. Five ml of an aqueous solution of apple uronides were reduced and degraded by the addition of .1 g Zn dust and heated for 15 hours at 55° C under H_2 pressure. Galacturonic, glucuronic acids and galactose, and glucose knowns

[3]Rohm and Haas; Philadelphia, Pennsylvania.

Table 2.—Degradation products of Golden Delicious, Stayman, and York Imperial apple AIS fraction and their avearge Rg values as compared with the standard sugars.

Standard sugars	Rg	Apple AIS degradation products	Rg
—	—	U[a]	11.7
D-galacturonic acid	16.5	+	16.4
D-glucuronic acid	22.5	+	21.4
—	—	U	45.4
—	—	U	59.9
D-galactose	90.6	+	90.2
D-glucose	100.0	+	100.0
L-arabinose	110.0	+	110.0
D-xylose	126.0	+	128.0

[a]Unknown.

were also treated with Zn dust, heat, and H_2 pressure. Resultant solutions were filtered and chromatogrammed as described under Paper Chromatography.

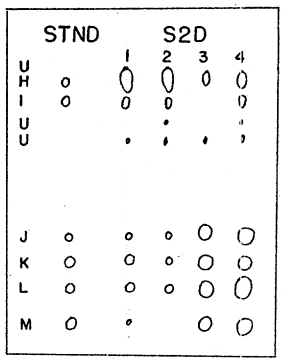
Results

In this study, several individual experiments were conducted. In the first experiment, the enzymatic break down products of the apple AIS fraction, i.e., 1) water soluble materials, 2) AO-OA soluble substances, 3) hemicellulose and 4) cellulose, were identified by comparing these substances with standard reference sugars. The color produced and the Rg values of these sugars were the indices used for identification. Table 2 gives the hydrolytic products for the 4 fractions studied and their average Rg values. D-galacturonic acid, D-glucuronic acid, D-galactose, D-glucose, and L-arabinose, and 3 unidentified substances were present in the water soluble and AO-OA extracted materials.

Because of the difficulty in completely separating galacturonic and glucuronic acids on the chromatograms, the apple hexuronides were partially reduced to galactose and glucose and further degraded to lyxose (or xylose) and arabinose. Approximately equal amounts of glucose and arabinose as well as galactose and lyxose were found. Degraded galactose gave lyxose and similarily treated glucose gave arabinose. This is evidence that apple hexuronides contain a mixture of galacturonic and glucuronic acids. Fig. 2 shows that the hemicellulose fraction contained all sugars listed above except glucuronic acid and the unknowns (Rg 45.4).[4] In the cellulose and hemicellulose fractions, xylose was also present. Fig. 2 shows an example of the hydrolytic products from each of these 4 fractions for the Stayman variety. They were similar for Golden Delicious, and York Imperial.

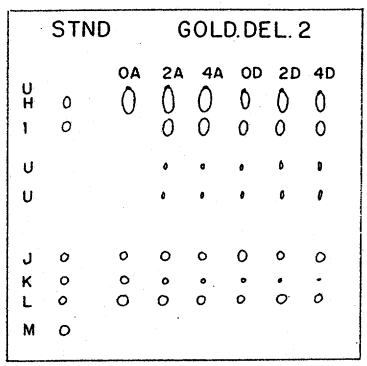
In all chromatograms the uronides and hexose sugars gave reddish brown spots, and the pentose sugars reddish, pink spots. The uronides were the main constituents of the water soluble and AO-OA extracted materials, (Fig. 2) indicating some selectivity in ex-

[4]Rg value is the ratio of distance traveled by a particular substance to that traveled by glucose X100.



U Unknowns
H D-galacturonic acid
I D-glucuronic acid
J D-galactose
K D-glucose
L L-arabinose
M D-xylose

Fig. 2. Paper chromatograms showing the hydrolytic products of: 1) water soluble materials, 2) ammonium oxalate-oxalic acid extracted substances, 3) hemicellulose, and 4) cellulose obtained from Stayman (S), 2 months storage (2), late harvest (D), apple.



U Unknowns
H D-galacturonic acid
I D-glucuronic acid
J D-galactose
K D-glucose
L L-arabinose
M D-xylose

Fig. 3. Paper chromatograms showing the ammonium oxalate-oxalic acid soluble degradation products obtained from early (A) and late (D) harvested Golden Delicious apples with 0, 2, and 4-month storage periods.

traction. However, in the cellulose and hemicellulose fractions, the hexuronic acids were of smaller proportion and lower color intensity when compared to the hexose and pentose sugars.

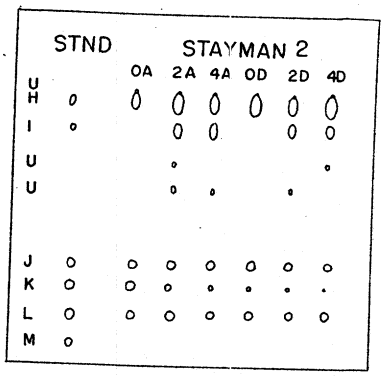
In another experiment, volumes of hydrolysates were standardized and equal amounts were placed on chromatograms for both the AO-OA and hemicellulose fraction. Figs. 3, 4 and 5 show that each of the 3 varieties studied contained the same group of monosaccharide hydrolysates in the AO-OA fraction, although they were very different in textural characteristics. This fraction was considered a "pectic substance" because of the predominance of the uronides.

Glucuronic acid did not appear in early harvested Golden Delicious and Stayman at harvest (Figs. 3, 4). However, it did appear in later harvests and in storage fruit from all other harvests of these varieties and also in the York Imperial (Fig. 5).

Glucose was found in all chromatograms, however, the size of the glucose spot decreased in later harvests and in fruit held in storage.

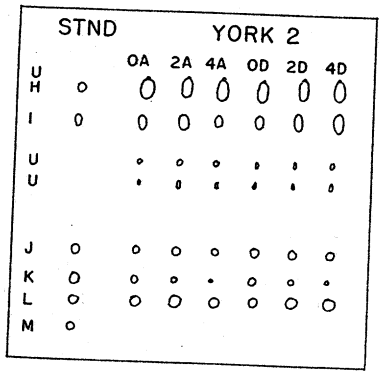
Although these analyses were on samples of fruit of widely varying firmness, noticeable differences in concentration and size of spots were found only in glucuronic acid and glucose.

In the hemicellulose fraction (Fraction 3) the uronides were a



U Unknowns
H D-galacturonic acid
I D-glucuronic acid
J D-galactose
K D-glucose
L L-arabinose
M D-xylose

Fig. 4. Paper chromatograms showing the ammonium oxalate-oxalic acid soluble degradation products obtained from early (A) and late (D) harvested Stayman apples with 0, 2, and 4-month storage periods.



U Unknowns
H D-galacturonic acid
I D-glucuronic acid
J D-galactose
K D-glucose
L L-arabinose
M D-xylose

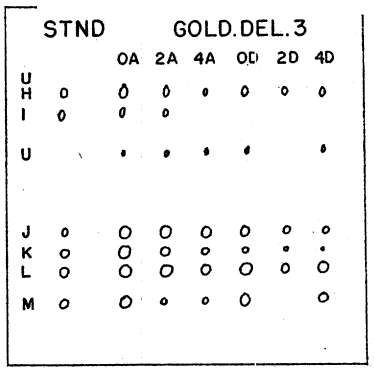
Fig. 5. Paper chromatograms showing the ammonium oxalate-oxalic acid soluble degradation products obtained from early (A) and late (D) harvested York Imperial apples with 0, 2, and 4-month storage periods.

smaller proportion of the whole as shown in Fig. 6 for Golden Delicious. Part of this difference was due to the absence of glucuronic acid in this fraction. Stayman and York Imperial chromatograms were very similar to Fig. 6. A decrease in glucose was noticeable in the late harvested fruit, and in samples that were riper.

Unknowns: Three unidentified substances have been found in apple degradation products. One of the unknown materials appeared as a relatively small pink spot, (Rg 11.7) just adjacent to galacturonic acid. The other unknowns (Rg 45.4 and 59.9) were found as separated light, brown spots, or as long streaked lines about the same color and located between the glucuronic acid and the galactose sugar spots. The unidentified pink spot, which ran very close to galacturonic acid, showed greater intensities and larger spots in the early harvested fruit. Fig. 7 shows this unknown material (top spot) which was separated from the galacturonic acid, only after lengthy irrigation. A spectrophotometric study of these substances in the ultra-violet, showed a peak at 299 mμ, for the uronide phase and at 314 mμ for the unknown pink material.

Discussion

The results reported here are similar to those of Jermyn and Isherwood (4) on Conference pear in which they found galacturonic acid, galactose, glucose, arabinose, xylose, mannose and rhamnose in the



U Unknowns
H D-galacturonic acid
I D-glucuronic acid
J D-galactose
K D-glucose
L L-arabinose
M D-xylose

Fig. 6. Paper chromatograms showing the hemicellulose degradation products obtained from early (A) and late (D) harvested Golden Delicious apples with 0, 2, and 4-month storage periods.

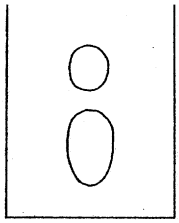


Fig. 7. Paper chromatogram showing the unknown (upper) and galacturonic acid (lower) spot.

acid hydrolysates of the cell-wall. The enzymatic break down of apple cell-wall material by enzymes was considered to be milder than acid hydrolysis and thus might account for finding glucuronic acid and other unidentified substances in this tissue.

Measurement of glucuronic acid and its separation from galacturontic acid were difficult since both followed closely on chromatograms and gave identical absorption curves in the ultra-violet. Both tended to form double spots on unwashed or streaks on acid washed papers.

Reduction and degradation of apple hexuronides into glucose and arabinose as well as galactose and lyxose in about equal amounts suggested substantial amounts of glucuronic acid in the apple cell-wall. The galacturonic acid portion of the apple uronide showed a greater tendency to degrade to a lower member than did the glucuronic acid.

Glucose spots which were found in the pectin and hemicellulose fractions in most harvests (maturities) decreased in color intensity and size as the fruits ripened. This glucose would not appear to be related to starch but is probably reduced glucuronic acid.

Chromatograms showed that the hemicellulose fraction, pentose-hexose oriented, decreased in color intensity and size of spot as the AIS was removed from softer and more mature fruit, indicating that this fraction may be involved in fruit softening.

The great admixture of monomers in the 4 fractions studied suggested that extraction procedures used in this work must be greatly modified to separate "pure" polysaccharides. In fact this may be nearly impossible no matter what extractants and time and temperature combinations are used.

This qualitative study of apple cell-wall monosaccharides should supply guidelines for more detailed studies of these substances and their related polysaccharides. It is possible that galacturonic and glucuronic acids must be separated and then related individually to changes in fruit firmness. It would also appear necessary to measure quantitatively the monosaccharides in the total apple AIS rather than measure either monosaccharides from individual fractions or measure only individual fractions based on selective extractions. When concentrations of the monosaccharides are ascertained it may be possible to evaluate the functional groups of each substance and relate them to apple texture.

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